Transketolase and 2',3'-Cyclic-nucleotide 3'-Phosphodiesterase Type I Isoforms Are Specifically Recognized by IgG Autoantibodies in

Transketolase and 2′,3′-Cyclic-nucleotide 3′-Phosphodiesterase Type I Isoforms Are Specifically Recognized by IgG Autoantibodies in Multiple Sclerosis Patients*[S]

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The presence of autoantibodies in multiple sclerosis (MuS) is well known, but their target antigens have not been clearly identified. In the present study, IgG autoreactivity to neural antigens of normal human white matter separated by bidimensional electrophoresis was assessed in serum and cerebrospinal fluid of 18 MuS and 20 control patients. Broad IgG autoreactivity was detected by two-dimensional immunoblotting in all cases to neural antigens, most of which were identified by mass spectrometry. The comparative analysis of MuS and non-MuS reactive spots showed that a restricted number of neural protein isoforms were specifically recognized by MuS IgG. Almost all MuS patients had cerebrospinal fluid IgG directed to isoforms of one of the oligodendroglial molecules, transketolase, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase type I, collapsin response mediator protein 2, and tubulin β4. Interestingly 50% of MuS IgG recognized transketolase, which was mostly localized on oligodendrocytes in human white matter from normal and MuS samples. IgG autoreactivity to cytoskeletal proteins (radixin, sirtuin 2, and actin-interacting protein 1) was prevalent in secondary progressive MuS patients. Among the proteins recognized by serum IgG, almost all MuS patients specifically recognized a restricted number of neuronal/cytoskeletal proteins, whereas 2′,3′-cyclic-nucleotide 3′-phosphodiesterase type I was the oligodendroglial antigen most frequently recognized (44%) by MuS seric IgG. Our immunomics approach shed new light on the autoimmune repertoire present in MuS patients revealing novel oligodendroglial and/or neuronal putative autoantigens with potential important pathogenic and diagnostic implications. ***Molecular & Cellular Proteomics 7: 2337–2349, 2008.***

Multiple sclerosis (MuS)[1] is an inflammatory disease of the central nervous system (CNS) in which the autoimmune response is directed to myelin and myelin-producing cells, the oligodendrocytes, and eventually leads to demyelination and oligodendrocyte loss (1). The autoimmune reaction has been shown to involve the activation of both T and B lymphocytes (2). The role of autoantibodies in MuS pathogenesis was suspected several years ago and has been confirmed recently by studying the autoreactivity of MuS sera to oligodendrocytes (3–5). In addition, accurate neuropathological studies have provided evidence that antibody-mediated autoimmune mechanisms contributed to lesion formation in a subset of MuS patients (6). Although MuS has long been regarded as a pure demyelinating disorder of the CNS, axonal degeneration was reported in early descriptions of MuS plaques and regained attention in recent years, now being recognized as an important pathological feature even in early MuS lesions and correlating with disease progression (7, 8). The mechanisms leading to axonal damage in MuS are largely unknown. Axonal damage is probably independent of demyelination (9), and a very recent study suggested that its pathogenesis is also autoantibody-mediated with neurofascin as a major autoantigen (10).

Despite extensive efforts, the molecular targets of the autoimmune response leading to demyelination in MuS have yet to be clearly defined. Among the myelin proteins investigated as potential targets for T or B cells, most studies have been focused on myelin basic protein (MBP), proteolipid lipoprotein (PLP), myelin oligodendrocyte glycoprotein (MOG), 2′,3′-cyclic-nucleotide 3′-phosphodiesterase type I (CNPase I), and synthetic peptides (11–14), whereas anti-cytoskeleton IgG

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[1] The abbreviations used are: MuS, multiple sclerosis; CNPase, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase; CRMP, collapsin response mediator protein; CSF, cerebrospinal fluid; CNS, central nervous system; HRP, horseradish peroxidase; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PP, primary progressive; RR, relapsing-remitting; SP, secondary progressive; PLP, proteolipid protein; PPP, pentose phosphate pathway; TK, transketolase; 2D, bidimensional.

TABLE I
Demographic and clinical features of MuS and control patients

M, male; F, female.

	Controls	Total MuS[a]	PP MuS[b]	SP MuS[c]	RR MuS[d]
Number	20	18	4	4	10
Age[e]	47.6±13.8 (22-71)	39.9±8.2 (29-59)	49.6±6.4 (44-59)	42.8±5.3 (38-52)	34.9±6.4 (29-48)
Sex ratio M/F	14/7	10/8	3/1	2/2	5/5
Disease duration (yr)[f]	N.D.[g]	6.2±5.4 (0.5-24)	5.6±2.9 (3-10)	11±8.2 (4-24)	4.5±3.2 (2-12)
EDSS[f]	N.D.	3.8±2.2 (1.5-9.5)	4.1±0.3 (4-4.5)	7.1±2 (5-9.5)	2.4±1.2 (1.5-5)

[a] MuS, multiple sclerosis.
[b] PP, primary progressive.
[c] SP, secondary progressive.
[d] RR, relapsing-remitting.
[e] All numbers are expressed as mean ± S.D. (range).
[f] At the moment of lumbar puncture.
[g] Expanded disability status scale evaluated at the moment of lumbar puncture.

has been associated with disease progression (10, 15, 16). To date, no MuS-specific biological markers have been identified (16). This may be due to the use of purified antigens and/or relevant peptides from preselected targets for the detection of autoantibodies in MuS patients. We have overcome such a restrictive approach by using a large panel of antigens derived from target tissue extracts. In the present study, we compared by bidimensional (2D) PAGE and immunoblotting the IgG repertoires from serum and cerebrospinal fluid (CSF) of control and MuS patients against antigens derived from CNS normal white matter. The reactive spots were then identified by MS and/or immunoblotting. This immunomics approach enabled the identification of a restricted number of neural protein isoforms specifically recognized by MuS sera and CSF that were mostly localized on oligodendrocytes and/or cytoskeleton. Besides the confirmation of anti-CNPase I IgG in MuS sera (13), the study of CSF IgG autoreactivity provided the most interesting information. Almost all MuS CSF displayed autoreactivity to oligodendroglial antigens, the most frequent of these being an isoform of transketolase (TK). In addition, autoreactivity to selected cytoskeletal proteins identified patients with secondary progressive MuS. Such newly identified antigenic targets, in addition to having important pathogenetic implications, may also serve as biomarkers of disease and could be useful diagnostic and prognostic tools.

MATERIALS AND METHODS

Patients—Sera and CSF were obtained for diagnostic purposes and after written informed consent from 38 subjects. Eighteen patients had clinically definite MuS with four primary progressive (PP), eight relapsing-remitting (RR), and four secondary progressive (SP) cases (17, 18); their clinical features are summarized in Table I. All MuS patients had CSF oligoclonal bands, were relapse-free, and had not received immunomodulating or immunosuppressive treatment in the previous 6 months. Control sera and CSF were obtained from 20 subjects affected with meningoencephalitis (4), myelitis (4), amyotrophic lateral sclerosis (3), neuropathy (3), and ischemic stroke (2). Four additional subjects underwent lumbar puncture to confirm meningitis but had spontaneous recovery and normal CSF biological parameters.

CNS Samples—Normal human white matter samples (Brodmann areas 4 and 6) for proteomics analysis were dissected out at autopsy from two healthy subjects with no history of neurological disease (one 37-year-old woman and one 56-year-old man; postmortem interval, 24 h), and the samples were frozen and stored at −80 °C. Autoptic CNS tissue from seven subjects (mean age, 51 years) with a clinical diagnosis of chronic progressive MuS was studied for immunohistochemical analysis. In total, 15 blocks containing lesions and normal appearing white matter were examined. Histopathologically three cases displayed a predominance of chronic active lesions, whereas in the remaining four cases the majority of lesions were chronic silent.

2D PAGE and Immunoblotting—White matter samples were homogenized in lysis buffer (7 M urea, 2 M thiourea, 0.4% CHAPS, 0.1% DTT, 0.5% Triton X-100) and subjected to high resolution 2D electrophoresis as reported previously (19). Isoelectrofocusing was carried out on non-linear immobilized pH gradients (pH 3–10; 18-cm-long IPG strips; GE Healthcare), and second dimension separation was carried out on 9–16% SDS-polyacrylamide gradient gels. The reference gel was stained with ammoniacal silver staining. For the 2D immunoblotting (20, 21), gels were blotted onto 0.2-μm nitrocellulose membranes that were subsequently blocked with 10% BSA and 0.1% Tween in TBS for 2 h and incubated with sera (diluted 1:10,000) or CSF (1:1,000) overnight. After washing, membranes were incubated with anti-human IgG horseradish peroxi-

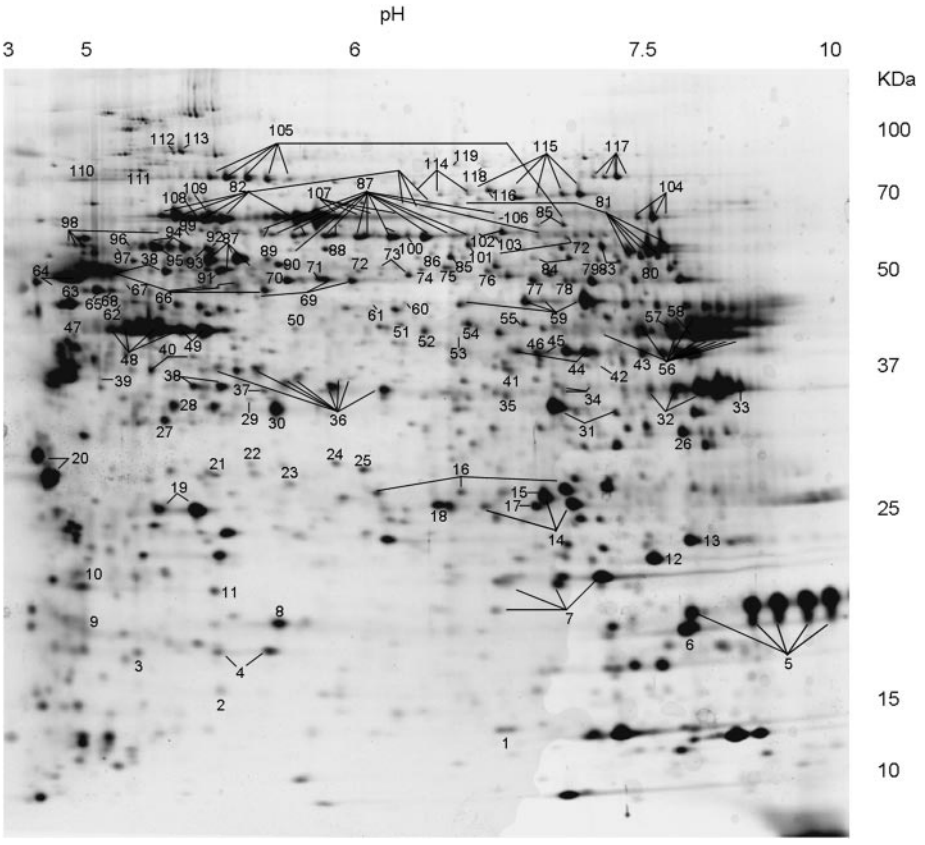


FIG. 1. **Protein map of the normal human white matter.** Normal human white matter was resolved by 2D PAGE, and the gel was subjected to silver staining; 243 of the spots recognized by IgG from MuS and controls were identified by MS and/or immunoblotting with specific antibodies and numbered from the low molecular masses and acidic pH to the high molecular masses and basic pH. The identity of each protein and its frequency of recognition by MuS IgG are indicated in Table II.

dase (HRP)-conjugated (1:3,000). Alternatively membranes were incubated with antibodies to the following proteins: collapsin response mediator proteins (CRMP) 5 (1:1,000) and 1 (1:30,000), radixin (1:1,000; Chemicon, Temecula, CA), α-centractin (1:50,000), NAD-dependent deacetylase sirtuin 2 (1:50,000; Abcam, Cambridge, UK), CNPase (1:20,000), actin (1:25,000), α-tubulin (1:35,000), neurofascin (1:2,500; Abcam), MOG (1:1,000; Sigma), MBP (1:50,000), glial fibrillary acidic protein (1:200,000; Dako, Milan, Italy), TK (1:1,000) (22), and TK 1 (1:400; kindly provided by J. F. Coy, R-Biopharm AG, Darmstadt, Germany). After incubation with the appropriate secondary HRP-conjugated Ig, the signal was developed with Advanced ECL (Amersham Biosciences). Immune profiles were analyzed when two independent assays had produced identical patterns. The reference 2D gel and autoradiographs were analyzed and matched with the Image Master 2D Platinum v6.0 software (Amersham Biosciences).

Immunoprecipitation—Specific recognition of TK and CNPase I by MuS CSF and serum was confirmed by testing the autoreactivity to immunoprecipitated TK and CNPase. For this purpose, 0.5 mg of CNS homogenate in buffer with 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA, 0.5% deoxycholic acid, pH 7.5, with protease inhibitors was incubated with anti-CNPase or -TK antibodies overnight at 4 °C. Protein A-agarose (Roche Applied Science) was then added for 3 h. After centrifugation, immunoprecipitated material was washed in cold TBS and extracted with lysis buffer. After addition of 2× Laemmli buffer, immunoprecipitated proteins were subjected to electrophoretic separation, blotted, and incubated with the respective antibodies. In parallel, immunoprecipitated CNPase was tested with four selected MuS sera (two of which recognized CNPase I by 2D immunoblotting), whereas immunoprecipitated TK was probed with four selected MuS CSF samples (two cases with anti-TK IgG reactivity).

Immunohistochemistry—Immunoperoxidase and double immunofluorescence was performed for the identification of the cellular localization of TK as described previously (5, 23). Briefly consecutive paraffin or frozen sections from normal human CNS and MuS cases were quenched and incubated with anti-TK antiserum and the following antibodies: CNPase, MBP, PLP (Chemicon), or NG2 (Sigma). Reactivity was revealed with the appropriate biotinylated antibody, avidin-biotin-HRP complex (Vector Laboratories, Burlingame, CA), and the reaction was visualized with 3′,3-diaminobenzidine as chromogen. In negative controls, primary antibodies were omitted. Selected sections were then counterstained with hematoxylin. To characterize the phenotype of glial cells expressing TK, double immunofluorescence was performed with glial phenotypic markers (14). For this purpose, TK was detected by anti-rabbit biotinylated antibody and avidin-Texas Red complex (Vector Laboratories), whereas the monoclonal antibodies to the glial markers CNPase, PLP, HLA-DR (Dako), NG2, and glial fibrillary acidic protein were identified with FITC-conjugated anti-mouse Ig.

In-gel Digestion and Protein Identification by Mass Spectrometry—Protein identification was carried out by peptide mass fingerprinting as described previously (24, 25). Electrophoretic spots, obtained from preparative 2D gels run with 1 mg of white matter homogenate, were visualized by an MS-compatible silver staining procedure (26, 27), manually excised, destained, and acetonitrile-dehydrated. They were then rehydrated in trypsin solution, and in-gel protein digestion was performed by overnight incubation at 37 °C. Each protein digest (0.75 μl) was spotted onto the MALDI target and allowed to air dry. Then 0.75 μl of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% (v/v) ACN and 0.5% (v/v) TFA) was applied to the dried sample and dried again. MS analysis was performed using an Ettan MALDI-TOF Pro mass spectrometer (GE Healthcare) in reflectron mode with an accelerating voltage of 20 kV. Mass spectra

TABLE II

Proteins of the normal human white matter identified

Periods in sequences represent cleavage sites. NSF, *N*-ethylmaleimide-sensitive factor; MAP, mitogen-activated protein kinase.

Nr.	ID	Name	Theoretical MW/pI	Experimental MW/pI	Identification score/ % coverage	Identification MS/MS (peptide sequence)	Immuno-blotting
1	P49773	Histidine triad nucleotide-binding protein 1	13670.7/ 6.46	13745/ 6.78	64/ 52	K.IIFEDDR.C	-
2	P61088	Ubiquitin-conjugating enzyme E2 N	17137.8/ 6.13	15334/ 5.46	126/ 32		-
3	P60983	Glia maturation factor β	16581.9/ 5.19	17061/ 5.16	86/ 35		-
4	P16949	Stathmin	17171.3/ 5.77	17149/ 5.64	97/ 31	R.ASGQAFELILSPR.S	-
5	Q59GX3	Myelin basic protein	22263/ 6.43	19600/ 8.11-10.54	-		+
6	P23528	Cofilin-1	18371.3/ 8.26	18265/ 8.03	76/ 49		-
7	P02511	α crystallin B chain	20158.9/ 6.76	21041/ 6.73-7.29	121/ 57		-
8	P00441	Superoxide dismutase [Cu-Zn]	15804.5/ 5.7	18549/ 5.66	117/ 54		-
9	P62760	Visinin-like protein 1	22011.1/ 5.01	18717/ 4.93	116/ 41		-
10	P02794	Ferritin heavy chain	21094.4/ 5.3	21204/ 4.09	188/ 59		-
11	P02792	Ferritin light chain	19888.4/ 5.51	20297/ 5.44	170/ 55		-
12	P30086	Phosphatidylethanolamine-binding protein 1	20925.5/ 7.43	22124/ 7.56	111/ 52		-
13	Q06830	Peroxiredoxin-1	22110.3/ 8.27	23292/ 8.08	128/ 40		-
14	P60174	Triosephosphate isomerase	26538.3/ 6.51	26251/ 6.68-7.14	131/ 38		-
15	Q16653	Myelin oligodendrocyte glycoprotein	25096.31/ 8.86	26455/ 7.00	-		+
16	P18669	Phosphoglycerate mutase 1	28672.7/ 6.75	26936/ 6.11-7.09	170/ 47	R.FSGWYDADLSPAGHEEAK.R	-
17	P09417	Dihydropteridine reductase	25803.5/ 6.9	25651/ 6.94	104/ 49		-
18	P30041	Peroxiredoxin-6	24903.7/ 6.02	25651/ 6.43	163/ 63		-
19	P09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1	24824.3/ 5.33	25355/ 5.37	167/51		-
20	P61981	14-3-3 protein γ	28171.4/ 4.8	28031/ 4.80	156/ 42		-
	P63104	14-3-3 protein ζ/δ		27531/ 4.78	128/ 44		-
	P62258	14-3-3 protein ε		27531/ 4.78	90/ 27		-
	Q04917	14-3-3 protein η		27531/ 4.78	81/ 29		-
21	P35232	Prohibitin	29804.1/ 5.57	28031/ 5.04	137/ 36		-
22	O95865	Dimethylarginine dimethylaminohydrolase 2	29643.8/ 5.66	28836/ 5.56	91/ 27		-
23	O95336	6-phosphogluconolactonase	27546.8/ 5.7	27284/ 5.7	91/ 30		-
24	Q9HOR4	Haloacid dehalogenase-like hydrolase domain	28535.9/ 5.84	28910/ 5.9	95/ 28		-
25	P78417	Glutathione transferase ω-1	27565.8/ 6.24	28358/ 6.04	67/ 20		-
26	P16152	Carbonyl reductase [NADPH] 1	30243.7/ 8.55	31400/ 7.96	89/ 26		-
27	Q9H115	Beta-soluble NSF attachment protein	33556.9/ 5.32	32500/ 5.27	143/ 52	K.VAAYAAQLEQYQK.A	-
28	P62873	Guanine nucleotide-binding protein subunit β 1	37245.7/ 5.6	34000/ 5.3	85/ 37	K.ACADATLSQITNNIDPVGR.I K.IYAMHWGTDSR.L R.ELAGHTGYLSCCR.F	-
29	P47755	F-actin capping protein α-2 subunit	32817.9/ 5.58	34570/ 5.56	100/ 36		-
30	P07195	L-lactate dehydrogenase B chain	36507.3/ 5.72	33519/ 5.65	154/ 36		-
31	P40925	Malate dehydrogenase	36294.9/ 6.89	33822/ 7.03	131/ 37	K.FVEGLPINDFSR.E	-
32	P04406	Glyceraldehyde-3-phosphate dehydrogenase	35922/ 8.58	35470/ 7.53-8.25	108/ 29		-
33	P04406	Glyceraldehyde-3-phosphate dehydrogenase	36070/ 8.58	35608/ 8.96	95/ 35		-
	P40926	Malate dehydrogenase, mitochondrial precursor	35595/ 8.92	35608/ 8.96	90/ 35		-
34	P15121	Aldose reductase	35722.2/ 6.55	35300-35800/ 7.1	163/ 37		-
35	Q9UBQ7	Glyoxylate reductase/hydroxypyruvate reductase	35668.2/ 7.01	34838/ 6.79	94/ 33	R.GDVVNQDDLYQALASGK.I	-
	O43488	Aflatoxin B1 aldehyde reductase member 2	39588.9/ 6.7	34838/ 6.79	90/ 25	R.VASVLGTMEMGR.R	-
36	Q8IXJ6	NAD-dependent deacetylase sirtuin-2	43182.1/ 5.22	35426-37682/ 5.45-6.15	260/ 68	R.LLDELTLEGVAR.Y	+
37	P37837	Transaldolase	37540/ 6.36	35289/ 5.62	76/ 25	K.LLGELLQDNAK.L	-
38	O94760	Dimethylarginine dimethylaminohydrolase 1	31313/ 5.53	35800/ 5.4	157/ 45	K.DENATLDGGDVLFTGR.E	+
39	Q14894	μ-crystallin homolog	33775.5/ 5.06	36582/ 5.00	132/ 42	K.FADTVQGEVR.V R.TAAVSAIATK.F	-
40	P09471	Guanine nucleotide-binding protein G(o), subunit α 1	39919.3/ 5.34	37500/ 5.22	96/ 21		-
	P29777	Guanine nucleotide-binding protein G(o), subunit α 2	39955.4/ 5.62	38800/ 5.37	95/ 21		-
41	P14550	Alcohol dehydrogenase[NADP+]	36441.8/ 6.35	37488/ 6.81	154/ 40		+
42	O43488	Aflatoxin B1 aldehyde reductase member 2	39588.9/ 6.7	37925/ 7.27	90/ 25		-
43	P04075	Fructose-bisphosphate aldolase A	39288.8/ 8.39	39215/ 7.5	70/ 24		-
44	P09972	Fructose-bisphosphate aldolase C	39324.6/ 6.46	39350/ 6.85-7.2	152/ 43		-
45	P09972	Fructose-bisphosphate aldolase C	39324.6/ 6.46	39316/ 7.00	75/ 29	R.TPSALAILENANVLAR.Y	-
	O00154	Brain acyl-CoA hydrolase	41796/ 8.85	39316/ 7.00	62/ 27	K. MIEEAGAIISTR	-
46	P28482	Mitogen-activated protein kinase 1	41258.5/ 6.53	39316/ 6.94	150/ 29		-
47	P14136	Glial fibrillary acidic protein	49880.2/ 5.42	41300-50090/ 4.9-5.12	120/29	K.LADVYQAELR.E	+
48	P60709	β-actin	41605.5/ 5.29	41700/ 5.00-5.30	133/ 36		+
	P63261	γ-actin	41661.6/ 5.31	41700/ 5.00-5.30	133/ 36		+
49	P12277	Creatine kinase B-type	42644.2/ 5.34	41831/5.4	210/ 58		-
50	P60709	β-Actin	41605.5/ 5.29	42209/ 5.73	58/ 20		+
	Q9P2R7-01	Succinyl-CoA synthetase, betaA chain	44584.7/ 5.61	42209/ 5.73	80/ 19		-
51	P42025	β-centractin	42293.4/ 5.98	42822/ 6.24	88/ 25		-

TABLE II—*continued*

Nr.	ID	Name	Theoretical MW/pI	Experimental MW/pI	Identification score/ % coverage	Identification MS/MS (peptide sequence)	Immuno-blotting
52	Q02750	MAP kinase kinase 1	43307.8/ 6.2	41755/ 6.36	70/ 21		-
53	P27361	Mitogen-activated protein kinase 3	43135.5/ 6.28	41400/ 6.54	149/ 33		-
54	P61163	α-centractin	42613.7/ 6.19	42900/ 6.6	141/ 39		+
55	P49411	Elongation factor Tu	45045/ 6.31	42900/ 6.88	221/ 46		-
56	P09543-01	2',3'-cyclic-nucleotide 3'-phosphodiesterase I	47578.6/ 9.17	41200-43900/ 7.5-9.3	196/ 29	K.APGAEEYAQQDVLKK.S	+
57	P00558	Phosphoglycerate kinase 1	44483.4/ 8.30	41200/ 8.2	104/28	K.ACANP AAGSVILLEN LR.F	-
58	P22695	Ubiquinol-cytochrome-c reductase complex	46784/ 7.74	42209/ 7.7	82/ 19	R.NALANPLYCPDYR.I	-
59	P06733	α-enolase	47037.7/ 6.99	47000/ 6.55-7.2	165/ 45		-
60	Q9NVA2	Septin-11	49267/ 6.38	46700/ 6.26	111/ 26		-
61	P31153	Methionine adenosyltransferase 2	43660.6/ 6.02	46700/ 6.09	93/ 29		-
	Q9NVA2	Septin-11	49267/ 6.38	46700/ 6.26	74/21		-
62	P07196	68 kDa neurofilament protein	61385.4/ 4.64	46700/ 5.10	79/ 19	R.EYQDLLNVK.M	-
63	P09104	γ-enolase	47137.3/ 4.91	46400/ 4.88	239/ 58		-
64	P68371	Tubulin β-2 chain	49831/ 4.79	51000/ 4.88	253/ 52	K.EVDEQMLNVQNK.N	-
65	P06576	ATP synthase subunit β	51769.2/ 5	48900/ 4.99-5.6	217/ 43		-
66	Q9BQE3	Tubulin α-6 chain	49895.3/ 4.96	52800/ 5.05-5.06	180/ 46	R.AVFVDLEPTVIDEVR.T	+
	Q71U36	Tubulin α-3 chain	50135.6/ 4.94	53300/ 5.00-5.06	178/ 45		+
	P68363	Tubulin α-ubiquitous chain	50151.6/ 4.94	53350-57650/ 4.96	178/ 45		+
67	P06576	ATP synthase subunit β	51769.2/ 5	50100/5.12	117/ 39	R.VALTGLTVAEYFR.D	-
	P04350	Tubulin β-4 chain	49585.7/ 4.78	50000/ 5.12	63/ 15	K.LAVNMVPFPR.L	-
	P14136	Glial fibrillary acidic protein	49880.2/ 5.42	50000/ 5.12	110/ 28	K.LADVYQAELR.E	+
68	P51854	Transketolase 1 (TKTL1)	65333.4/ 5.56	49000/ 5.06	-		+
69	Q92599	Septin-8	55625/ 5.89	47700-50700/ 5.59-5.98	226/ 44		-
70	Q96KP4	Glutamate carboxypeptidase-like protein 1	52878.4/ 5.66	50818/ 5.69	65/ 14		-
71	P05091	Aldehyde dehydrogenase class 2	54444.9/ 5.69	51093/ 5.81	133/ 22		-
72	Q14194	Collapsin response mediator protein 1	62183.8/ 6.55	52777-60581/ 6.02-7.1	192/ 24		+
73	P78371	T-complex protein 1 subunit β	57357/ 6.02	52100-53180/ 6.14-6.28	114/ 27		-
74	P55809	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1	52089.8/ 6	53000/ 6.35	156/ 35		-
75	O43175	D-3-phosphoglycerate dehydrogenase	56519.3/ 6.31	53350/ 6.47	112/ 21		-
76	P00352	Aldehyde dehydrogenase family 1 member A1	54730.6/ 6.29	52777/ 6.69	89/ 21		-
77	Q16658	Fascin	54398.8/ 6.81	50900/ 6.92	252/ 51		-
78	Q16658	Fascin	54398.8/ 6.81	50900/ 7.00	90/ 27		-
	P00367	Glutamate dehydrogenase 1	56008.6/ 6.71	50900/ 7.00	96/ 21		-
79	P00367	Glutamate dehydrogenase 1	56008.6/ 6.71	50635/ 7.23	203/ 38		-
80	O00231	26S proteasome non-ATPase regulatory subunit11	47332.5/ 6.09	54600/ 7.52	77/ 22		-
81	P14618	Pyruvate kinase isozymes M1/M2	57805.7/ 7.95	55800-58200/ 7.49-7.68	219/ 42	R.NTGIICTIGPASR.S	-
82	P11142	Heat shock 70 kDa protein 1, 2, 8, 12A	70038-70898/ 5.37-5.56	66032-67400/ 5.26-5.69	271/ 50		-
83	P30038	Aldehyde dehydrogenase 4A1	59034.1/ 6.96	54714-57900/ 7.28-7.38	107/ 17		-
84	Q9BPU6	Collapsin response mediator protein 5	61421.3/ 6.73	55400/ 6.93-7.12	120/ 17		-
85	P61764	Syntaxin-binding protein 1	67568.7/ 6.5	55509-64300/ 6.5-7.08	151/ 19		-
86	P49591	Seryl-tRNA synthetase	58646/ 6.06	56315/ 6.40	118/ 25		-
87	Q16555	Collapsin response mediator protein 2	62293.6/ 5.95	50818-62484/ 5.36-6.94	319/ 60	R.GLYDGPVCEVSVTPK.T	+
88	Q16555	Collapsin response mediator protein 2	62293.6/ 5.95	57400/ 5.84	143/37		+
	P17987	T-complex protein 1 subunit α	60343.5/ 5.8	57400/ 5.84	100/ 37		-
89	Q16555	Collapsin response mediator protein 2	62293.6/ 5.95	55200/ 5.63	108/ 26		+
	Q14195	Collapsin response mediator protein 4	61963.3/ 6.04	55200/ 5.63	250/ 49		-
90	Q16555	Collapsin response mediator protein 2	62293.6/ 5.95	54223/ 5.67	107/ 25		+
	P30101	Protein disulfide-isomerase A3 precursor	54265.2/ 5.61	54223/ 5.67	123/ 25		-
91	P21281	Vacuolar ATP synthase subunit B, brain isoform	56500.7/ 5.57	52400/ 5.45	120/ 38		-
	P07197	160 kDa neurofilament protein	102316.8/ 4.9	52400/ 5.45	69/ 20		-
92	P50990	T-complex protein 1 subunit τ	59489.3/ 5.42	56900/5.43	158/ 24		-
93	P48643	T-complex protein 1 subunit ε	59671/ 5.45	57442/ 5.42	178/ 33		-
94	Q16352	66 kDa neurofilament protein	55390.6/ 5.34	57900/ 5.3	271/ 46		-
95	P10809	Heat shock protein 60 kDa	57962.8/ 5.24	57900/ 5.24	136/ 29		-
96	P10809	Heat shock protein 60 kDa	57962.8/ 5.24	57650/ 5.13	86/ 30		-
	P68363	Tubulin α-ubiquitous chain	50804/ 4.94	57650/ 5.13	122/ 42		+
97	P68363	Tubulin α-ubiquitous chain	50804/ 4.94	57650/ 5.08	170/ 40		+
98	O00499	Myc box-dependent-interacting protein 1	64699.3/ 4.97	58400-62800/4.86-5.25	124/ 33	R.GVFPENFTER.V	-
99	P48643	T-complex protein 1 subunit ε	59671/ 5.45	61250/ 5.36	116/ 19		-
100	P49368	T-complex protein 1 subunit γ	60533.8/ 6.1	60983/ 6.28	91/ 21		-
101	P40227	T-complex protein 1 subunit ζ	57892.9/ 6.25	58347/ 6.59	126/ 21		-
102	O75083	Actin-interacting protein 1	66062.3/ 6.18	61800-63000/ 6.7	165/ 28	R.FATASADGQIYIYDGK.T	-
103	P31939	Bifunctional purine biosynthesis protein	64615.8/ 6.27	61400/ 6.7	151/ 25		-
104	P29401	Transketolase	67877.6/ 7.58	66700/ 7.46-7.68	199/ 36	K.ILATPPQEDAPVV SVDIANIR.M	+
105	P06396	Actin-depolymerizing factor	82959.1/ 5.72	82950/ 5.42-5.6	210/ 30	K.AGALNSNDAFVLK.T	-

TABLE II—*continued*

Nr.	ID	Name	Theoretical MW/pI	Experimental MW/pI	Identification score/ % coverage	Identification MS/MS (peptide sequence)	Immuno-blotting
106	P31040	Succinate dehydrogenase	68012/ 6.25	66200/ 6.73	112/ 15		-
107	P02768	Human serum albumin	66472.2/ 5.67	66900/ 5.9-6.2	164/ 29		-
108	P08133	Annexin A6	75742/ 5.42	66900/ 5.4	130/ 25		-
	P11142	Heat shock 70 kDa protein 8	70898.0/ 5.37	66900/ 5.4	79/ 19		-
109	P08133	Annexin A6	75742/ 5.42	68400/ 5.42	232/ 41		-
	P38646	75 kDa glucose regulated protein	68759/ 5.44	68000/ 5.42	59/ 15		-
110	P07900	Heat shock protein 90 α	84528.5/ 4.94	82000/ 4.94	188/ 29		-
111	P55072	Transitional endoplasmic reticulum ATPase	89190.6/ 5.14	86800/ 5.17	116/ 19		-
112	Q14764	Major vault protein	99195.7/ 5.34	99000/ 5.30	95/ 13		-
113	P49588	Alanyl-tRNA synthetase	106801.4/ 5.31	99000/ 5.33	123/ 17		-
	P07197	160 kDa neurofilament protein	102316.8/ 4.9	99000/ 5.33	69/ 20		-
114	P35241	Radixin	68563.9/ 6.03	73600/ 6.3-6.58	124/ 16		+
115	P02787	Transferrin	75181.4/ 6.7	74000/ 6.58-7.17	253/ 35		-
116	P46459	Vesicle-fusing ATPase	82654.3/ 6.38	76000/ 6.69	105/ 17		-
117	Q99798	Aconitase	82425.7/ 6.85	83200/ 7.25-7.42	228/ 31		-
118	P21399	Iron-responsive element-binding protein 1	98398.8/ 6.23	90000/ 6.67	168/ 20		-
119	Q05193	Dynamin-1	97745/ 6.93	90300/ 6.53	118/ 13		

were acquired automatically using the Ettan MALDI Evaluation software (GE Healthcare). Spectra were internally calibrated using the autoproteolysis peptides of trypsin (842.51 and 2211.10 Da). All the resulting mass lists were automatically cleaned up from contaminant masses, such as those from matrix and autodigestion of trypsin and keratins. All the spectra with the corresponding monoisotopic mass list are presented in supplemental Document 1. Mass fingerprinting searching was carried out in Swiss-Prot/TrEMBL 48.9 and NCBInr 20071013 databases using MASCOT (Matrix Science Ltd., London, UK) on line-available software. The taxonomy was limited to *Homo sapiens*, a mass tolerance of 100 ppm was allowed, and the number of accepted missed cleavage sites was set to 1. Alkylation of cysteine by carbamidomethylation was assumed as fixed modification, whereas oxidation of methionine was considered as a possible modification. The experimental mass values were monoisotopic. No restrictions on protein molecular weight and pI were applied. The criteria used to accept identifications included the extent of sequence coverage, number of matched peptides, and probabilistic score sorted by the software as detailed in Table II. Tryptic digests that did not produce MALDI-TOF unambiguous identifications were subsequently acidified with 2 μl of a 1% trifluoroacetic acid solution and then subjected to ESI-ion trap MS/MS peptide sequencing on a nanospray/LCQ DECA ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). Using ZipTip^{TM} pipette tips for sample preparation (Millipore, Billerica, MA) previously equilibrated in 50% acetonitrile solution and abundantly washed in 0.1% trifluoroacetic acid, acidified samples were enriched. Tryptic peptide elution from the ZipTip matrix was achieved with a 70% methanol and 0.5% formic acid solution, and 3 μl of such concentrated sample solutions were then loaded in a distal coated fused silica Picotip emitter to be nanosprayed in the mass spectrometer. The collision energy was set based on the mass of the precursor ions, which are doubly charged, and spectra were acquired using Excalibur software (Thermo). All the MS/MS spectra are provided in supplemental document 2, and identified peptides are provided in Table II. MS/MS database searching was performed by TurboSEQUEST (Thermo) and Mascot MS/MS ion search software (Matrix Science) in the Swiss-Prot/TrEMBL 48.9 or NCBInr 20071013 databases. The following criteria were applied: MS accuracy, $\pm$1.2 Da, MS/MS mass accuracy, $\pm$0.6 Da, peptide precursor charge, 2+, monoisotopic experimental mass values, trypsin digestion with one allowed missed cleavage, fixed carbamidomethylation of cysteine, and variable oxidation of methionine.

Statistical Analysis—To perform the analysis of IgG autoreactivity using χ^2 or Fisher exact test, the data were expressed in binary mode (0 = absence and 1 = presence of immune reactive spot in all the subjects tested). This descriptive approach allowed us to select the most relevant spots in the serum and in the CSF, which supported qualitatively different immune recognition between control and MuS patients. In a second stage, all the reactive spots present exclusively in MuS patients and having a p value <0.05 in the previous statistical tests were considered alone or grouped according to their cellular localization (*i.e.* proteins known to be present in cytoskeletal/neuronal, oligodendroglial, or both compartments). Comparison of the number of spots between MuS and control groups (either in serum or CSF) was performed by Wilcoxon rank-sum test. Comparison between the number of spots and disease duration was performed with Spearman rank correlation. To evaluate whether MuS clinical subgroups had different IgG autoreactivity to functional groups of proteins, the number of reactive spots for each group was calculated in all MuS cases; successively the Kruskal-Wallis test was used to evaluate such scores in each MuS clinical subgroup.

RESULTS

Bidimensional electrophoretic separation of human white matter proteins showed about 1,500 spots after silver staining, 350 of which were recognized by IgG of at least one patient; the frequency of recognition of each spot is reported in supplemental Table 1. By MS and/or immunoblotting, we identified 243 of these spots (69%), which corresponded to 120 distinct proteins (Fig. 1 and Table II); as already described for rodent CNS (28), several isoforms were detectable for most of these proteins. Among the spots identified, those recognized exclusively and with high frequency by MuS IgG were almost all included (supplemental Table 1); thus, this methodological approach provided an exhaustive scenario of white matter antigens recognized by autoantibodies present in serum and CSF of all the patients examined.

CSF IgG Autoreactivity—No difference in the number of spots recognized by CSF IgG was observed between MuS

TABLE III
IgG autoreactivity to statistically relevant white matter proteins in MuS and control patients

CSF[a]				
Accession Number	Nr in Fig. 1	Protein	Frequency (%) in MuS	Frequency (%) in Controls
Q8IXJ6	36	Sirtuin 2	16.7[b]	0
P09543	56	CNPase I	22.2	0
P07196	62	68 kDa neurofilament protein	38.9	5.0
P04350	67	Tubulin β4	16.7	0
P55809	74	Succinyl-CoA ketoacid- transferase	72.2	35.0
Q16555	87	CRMP2	33.3	0
O75083	102	Actin interacting protein 1	16.7	0
P29401	104	Transketolase	50.0	0
P35241	114	Radixin	16.7	0
Serum				
Accession Number	Nr in Fig. 1	Protein	Frequency (%) in MuS	Frequency (%) in Controls
P09972	44	aldolase C	50.0[a]	20.0
P61163	54	α-centractin	22.2	0
P09543	56	CNPase I	44.4	0
Q14194	72	CRMP1	16.7	0
P55809	76	Succinyl-CoA ketoacid- transferase	50.0	10.0
P14618	81	Pyruvate kinase	22.2	0
P30038	83	Aldehyde dehydrogenase 4A1	33.3	5.0
Q9BPU6	84	CRMP5	16.7	0
P61764	85	Syntaxin binding protein 1	44.4	0
P29401	104	Transketolase	22.2	0

[a] Cerebrospinal fluid.
[b] $p < 0.05$ in comparison with control patients.

(mean ± S.D., 60.9 ± 15.4; median, 63) and control groups (mean ± S.D., 62.9 ± 18.3; median, 66). The frequency of recognition of each reactive spot is listed in supplemental Table 1. Table III summarizes the protein isoforms recognized with higher frequency by CSF IgG from MuS patients as compared with controls. All MuS patients displayed CSF reactivity to at least one of these molecules. Among these, of particular interest were those recognized exclusively by MuS patients. All but one MuS patient reacted with at least one isoform of TK, CNPase I, radixin, CRMP2, tubulin β4, NAD-dependent deacetylase sirtuin 2, or actin-interacting protein 1 (Table IV and supplemental Fig. 1). As shown in Figs. 2 and 3, 2D PAGE separation of white matter antigens showed that most of these proteins exclusively recognized by MuS CSF existed in multiple isoforms. In addition, 2D immunoblotting provided

TABLE IV
IgG autoreactivity in MuS patients to MuS-specific white matter proteins grouped according to their cellular localization

CSF			
Cellular localization		Proteins	Frequency
1	Neurons/axons	Sirtuin 2, Radixin, Actin interacting protein 1	44.4
2	Oligodendrocytes	CNPase I, tubulin β4	33.3
3	Neurons/Oligodendrocytes	Transketolase, CRMP2	61.1
2 + 3			94.4
All MuS-specific proteins			94.4
Serum			
Cellular localization		Proteins	Frequency
1	Neurons/axons	α-centractin, CRMP1, Pyruvate kinase, Syntaxin binding protein 1	72.2
2	Oligodendrocytes	CNPase I	44.4
3	Neurons/Oligodendrocytes	Transketolase, CRMP5	33.3
2 + 3			61.1
All MuS-specific proteins			88.9

clear evidence that only single isoforms were specifically recognized by MuS IgG; the others also reacted with control sera. In this regard, Fig. 2 shows the paradigmatic examples of the recognition of TK and CRMP5 by CSF and serum MuS IgG, respectively. Transketolase is a protein of 67.8 kDa and pI 7.4–7.6, and the more acidic isoform was recognized by 50% of MuS CSF and by 22% of MuS sera but not by controls (Fig. 2, *A* and *B*, and Table III). Noteworthy is that such autoreactivity would have been missed by monodimensional immunoblotting because of the unspecific signals to the more basic TK isoforms (Fig. 2, *A* and *B*) as well as to other molecules with similar weight, such as albumin or heat shock proteins (Fig. 2*A*, *arrowheads*), which were recognized by most controls. Recognition of TK by CSF was confirmed by testing IgG from MuS and control CSF against immunoprecipitated TK; for this purpose, we selected two cases with reactivity to the MuS-specific isoform of TK and two control CSF samples without TK immunoreactivity by 2D immunoblotting. Monodimensional immunoblotting showed an immunoreactive band at the same molecular weight of TK only with MuS CSF (Fig. 2*C*). Interestingly when the molecules recognized by MuS CSF IgG were grouped according to their cellular distribution within the CNS, all but one MuS patient displayed IgG autoreactivity to antigens expressed on oligodendrocytes, such as CNPase I (29), tubulin β4 (30), TK (Ref. 31 and next paragraph), and CRMP2 (32, 33) (Table IV). IgG autoreactivity to the MuS-specific neuronal/axonal proteins radixin, sirtuin 2, and actin-interacting protein 1 (34–36) was present in a lower proportion of MuS CSF samples. Interestingly the number of cytoskeletal molecules recognized by CSF IgG was significantly higher in SP MuS (median, 1.25) than in PP

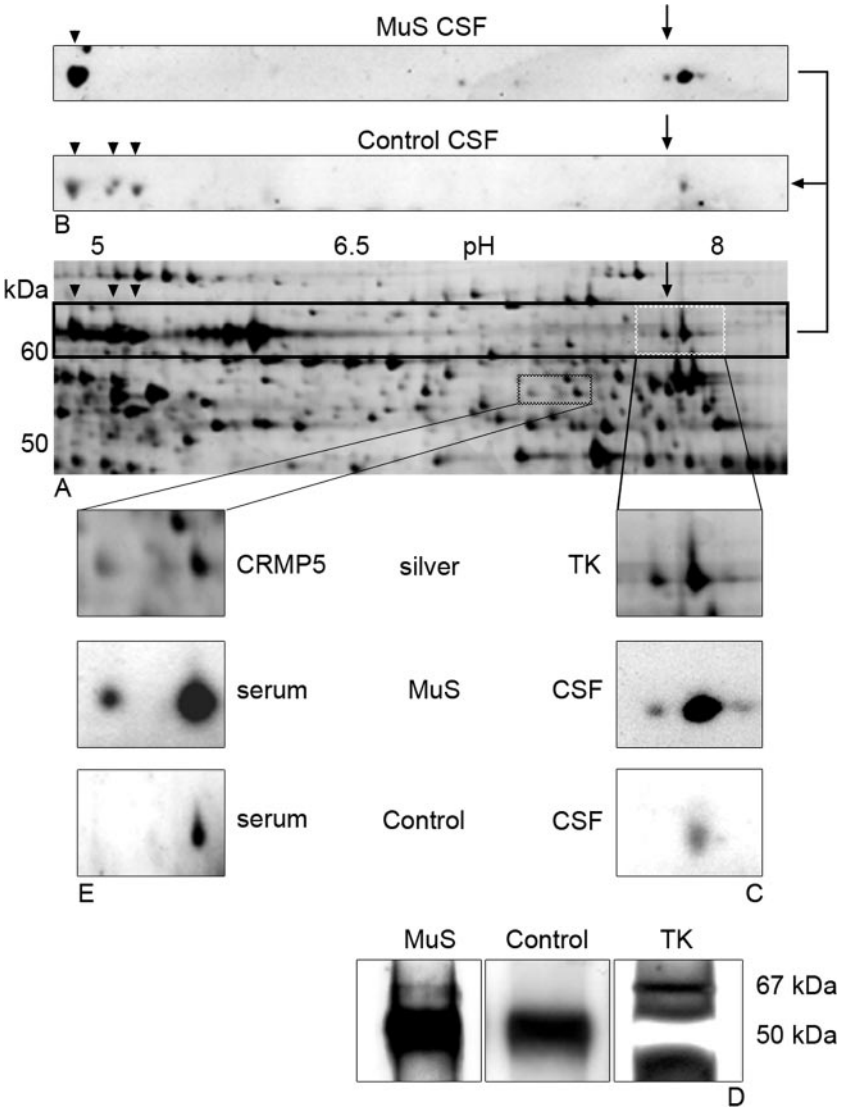


FIG. 2. **MuS-specific neural protein isoforms are selectively recognized by serum and CSF IgG from MuS patients.** Two examples of the advantages of 2D immunoblotting for the recognition of MuS-specific protein isoforms are shown. *A*, focusing on the neural proteins at 65–70 kDa in the silver-stained gel (*black rectangle*), it is evident that TK isoforms (*arrow*) have a mass similar to that of the series corresponding to heat shock proteins and albumin (*arrowheads*). *B* and *C*, pictures of 2D immunoblotting (of the same areas corresponding to the *rectangles* in *A*) of two membranes probed with MuS (*upper panel*) and control (*lower panel*) CSF. Immunoreactivity for the more acidic TK isoform (*arrow*) is exclusively present in MuS CSF. In addition, the signal of the MuS-specific TK isoform visible on 2D immunoblotting (*B*) is clearly distinguishable from heat shock proteins (*arrowhead*), whose reactivity is present in most patients. *D*, specificity of TK recognition by MuS CSF is confirmed by using immunoprecipitated TK. TK immunoreactivity is visible at 67 kDa with specific antiserum (*right lane*) and MuS CSF (*left lane*; selected among those with anti-TK reactivity) but not with CSF from controls without anti-TK autoantibodies (*central lane*); in all cases, a band corresponding to IgG (used for immunoprecipitation) is visible at 50 kDa. *E*, MuS-specific reactivity to one CRMP5 isoform in MuS serum; of the two CRMP5 isoforms identified in the silver-stained gel (*upper panel*), only the more acidic isoform is specifically recognized by MuS serum (*central panel*), whereas the other reacts also with control sera (*lower panel*).

(median, 0.25) or RR (median, 0) MuS cases ($p < 0.001$), and such difference was not related to different disease duration among MuS clinical subgroups (data not shown).

Cellular Localization of TK in Normal Human White Matter and MuS Lesions—Prompted by the selective and frequent recognition of TK by MuS IgG, we assessed its distribution in normal human white matter and MuS lesions by immunoperoxidase and double immunofluorescence with glial phenotypic markers. Transketolase is an enzyme of the pentose phosphate pathway (PPP), which has been described in neurons and oligodendrocytes in the rat brain (31). In normal human brain we found that TK was expressed on nearly all mature oligodendrocytes and scattered NG2-positive precursors, diffusely on ependymal cells, and on distinct subsets of neuronal populations (Fig. 4, *A–E*). In MuS sections, TK immunoreactivity was still present on oligodendrocytes in normal appearing white matter up to the edge of both active and silent lesions and markedly decreased in the core of demyelinated lesions (Fig. 4, *F* and *G*). The comparative analysis of the signals for TK and myelin proteins at the lesion border showed that immunoreactivity for TK was more extensive than myelin staining (Fig. 4, *F–H*). Double staining with glial phenotypic markers in this area demonstrated the expression of TK on subsets of NG2-positive oligodendrocyte precursors, reactive astrocytes, and monocyte/macrophages (data not shown). No major difference in terms of TK expression was observed upon neurons or ependymal cells in comparison with normal CNS samples (data not shown).

Serum IgG Autoreactivity—The number of spots reactive for serum IgG was higher in MuS patients (mean ± S.D., 60.8 ± 18.0; median, 62.5) as compared with control cases (mean ± S.D., 47.3 ± 22.6; median, 46; $p < 0.0065$). Regarding the reactivity to the white matter proteins identified, supplemental Table 1 shows the frequency of recognition of each spot, whereas Table III summarizes the molecules recognized with

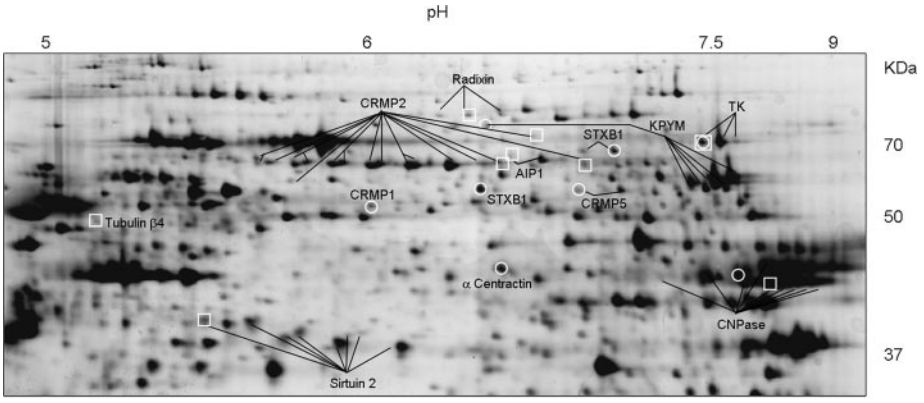
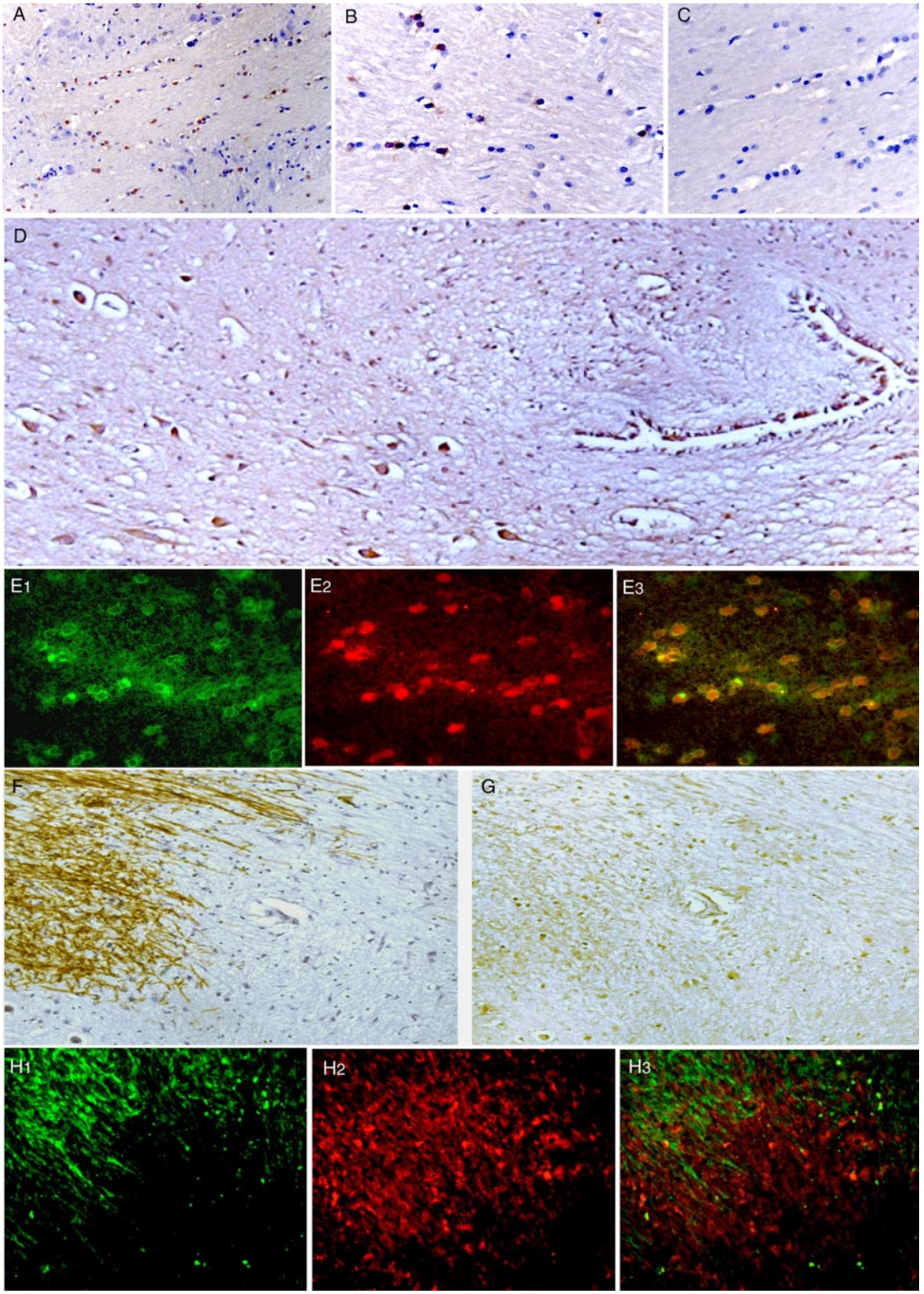


FIG. 3. **MuS-specific proteins of normal human white matter.** This figure summarizes the neural antigens recognized exclusively by MuS patients among the complex array of IgG autoreactivity. Each protein of interest is present in multiple isoforms, but MuS specificity is limited to one or two isoforms. The comparative analysis of the MuS-specific spots recognized by CSF (identified by *white squares*) and serum (*white circles*) IgG showed the importance of post-translational modifications of putative MuS autoantigens and the compartmentalization of the immune response in the blood and CSF with minimal overlap between the two groups of autoreactive proteins. *Sirtuin 2*, NAD-dependent deacetylase sirtuin 2; *STXB1*, syntaxin-binding protein 1; *AIP1*, actin-interacting protein 1; *KPYM*, pyruvate kinase.

higher frequency by serum IgG from MuS patients as compared with controls. Focusing on MuS-specific reactivity, we found that isoforms of CNPase I, α-centractin, CRMPs 5 and 1, syntaxin-binding protein 1, TK, and pyruvate kinase were recognized exclusively by IgG from MuS sera (Fig. 3 and Table III). All but two MuS sera displayed IgG reactivity to at least one of these proteins (Table IV). Similarly to that described for CSF reactivity, single isoforms of these proteins were recognized by serum IgG from MuS patients but not from control IgG (see the example of CRMP5 in Fig. 2*E*). Among MuS-specific molecules, the most frequently recognized from MuS sera were one isoform of CNPase I and two of syntaxin-binding protein 1 (Table III). Noteworthy is that 2D immunoblotting showed that different isoforms of CNPase I were recognized by IgG from serum and CSF of MuS patients (Fig. 3). Specific autoreactivity to CNPase was confirmed by immunoprecipitation (data not shown). When MuS-specific molecules were clustered according to their cellular distribution, 72% of MuS sera displayed autoreactivity to at least one of the neuronal proteins α-centractin, CRMP1, syntaxin-binding protein 1 (33, 37)- and pyruvate kinase, whereas about 60% of MuS patients had IgG reactivity to antigens localized (exclusively or not) on oligodendrocytes (Table IV). Serum IgG autoreactivity to any MuS-specific protein or group of proteins did not discriminate among MuS clinical subgroups (data not shown).

Comparative Analysis of IgG Reactivity in Serum and CSF—The comparative analysis of IgG autoreactivity to white matter antigens detectable in serum and CSF of MuS patients showed overall that different MuS-specific proteins or protein isoforms were recognized with high frequency in these two compartments (supplemental Table 1), and the results are summarized in Fig. 3. In fact, only in the case of TK, the 2D immunoblotting showed that both serum and CSF IgG from three MuS cases reacted with the same TK isoform. Instead different isoforms of CNPase I were recognized with high frequency by serum and CSF IgG in MuS (Fig. 3). As for the MuS-specific neuronal/axonal proteins, no autoreactivity was detected in the serum for those molecules recognized by CSF IgG (*i.e.* radixin, sirtuin 2, and actin-interacting protein 1). Thus, the overall autoreactivity to white matter antigens present in serum and CSF from MuS patients was directed to different proteins, a strong evidence for a compartmentalized autoimmune response. On the contrary, the comparative analysis in control patients showed a more general overlap between the neural proteins recognized by serum and CSF IgG with about 40–50% of spots reacting with IgG from both compartments.

IgG Autoreactivity to Other MuS-relevant Proteins—As for the IgG reactivity to other MuS-relevant proteins, we focused our attention on neurofascin (10), MBP (11, 12), MOG (11, 12), transaldolase (38), α-enolase (39), triose-phosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase (40). The present proteomics approach showed in general that IgG autoreactivity to these MuS-relevant molecules was present both in MuS and control patients. In fact, anti-transaldolase IgG was detected only in one MuS serum, whereas anti-MOG antibodies were observed in 65–90% of samples either in MuS and control cases (supplemental Figs. 1 and 2). Autoreactivity to MBP isoforms was present in 5–20% of all patients both in serum and CSF. In this regard, it would have been interesting to establish whether one or more of the MBP isoforms identified in our map were citrullinated because this post-translational modification has suggested novel mechanisms in the pathogenesis of MuS (41). Unfortunately we were not able to establish whether one or more of the MBP isoforms identified in our map were citrullinated because we identified this protein only by immunoblotting with a specific antiserum but not by mass spectrometry. The frequency of autoreactivity to α-enolase depended on the isoform consid-


A
B
C
D
E1
E2
E3
F
G
H1
H2
H3

ered, the more basic isoforms being recognized by most MuS and control patients (supplemental Figs. 1 and 2) and the other isoforms being recognized by 0–30% (both in serum and CSF). Similar findings were observed for triose-phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase whose more acidic isoforms were recognized by most MuS and control CSF and the others by 15–35% of cases (supplemental Figs. 1 and 2). Although we focused on a lower range of molecular masses, both series of neurofascin isoforms (155 and 186 kDa) were present in our homogenate and were recognized by 30–50% of both MuS and control patients. We failed to detect PLP in our human white matter map and were unable to assess any autoreactivity to this molecule.

DISCUSSION

In the present study, we evaluated the patterns of autoimmune recognition of human white matter antigens by the comparative analysis of autoreactive IgG repertoires present in serum and CSF from MuS and control patients. We used a bidimensional immunoblotting assay to develop a global approach against a large panel of natural antigens. A total of 350 spots were recognized by patients' IgG (either from serum or CSF), and the identification of two-thirds of these spots provided an exhaustive scenario of the autoreactivity of serum and CSF IgG. In comparison with previous studies, which addressed this topic by monodimensional immunoblotting (39, 42, 43), our bidimensional approach showed the existence of multiple molecules with similar molecular weights; as a consequence, immunoreactivity of these proteins would probably overlap, and the discrimination of the single reactivities could be challenging. In addition, bidimensional immunoblotting underscored the importance of post-translational modifications of neural proteins; in fact, only single isoforms of a given neural protein were specifically recognized by MuS patients and thus would be considered as MuS putative autoantigens. These lines of evidence highlight the limitation of the monodimensional approach to study antibody autoreactivity to CNS antigens. Despite the use of a high performance method, we are aware that the analysis of IgG autoreactivity to white matter antigens by (mono- or bidimensional) immunoblotting remains restricted. In fact, the electrophoretic procedure limits the analysis of autoantibody response to nonconformational epitopes and to proteins with sufficiently high levels of expression. With the limitation in mind that autoreactivity to low abundance proteins may have been underevaluated, our immunomics approach for the detection of IgG autoantibodies to neural antigens in MuS patients and the identification of the putative MuS autoantigens provided several innovative clues with pathogenic and diagnostic relevance for this disease. Our study confirmed that serum and CSF IgG from all patients recognized multiple antigenic spots of normal CNS homogenate resulting in a complex array of IgG autoreactivity to white matter protein isoforms so that distinct patterns were observed in each patient investigated (39). Within such heterogeneous autoreactivity, the comparative analysis of IgG repertoires of control and MuS patients revealed the presence of a number of protein isoforms recognized exclusively and with high frequency by MuS patients either in serum and CSF. As for MuS-specific autoantigens recognized by serum IgG, the majority of MuS patients displayed autoreactivity to at least one protein isoform localized in the neuronal/cytoskeletal compartment. Among oligodendroglial molecules, serum autoantibodies to an isoform of CNPase I were present in 44% of MuS patients. Such autoreactivity has already been reported in MuS patients using a similar proteomics approach (13); at variance with our results where anti-CNPase I IgG recognized only a specific isoform, in that study IgM but not IgG was found to react to all CNPase I isoforms. Such a difference is probably related to different technical conditions (such as serum dilution and sensitivity of detection). Nevertheless both studies showed the presence of anti-CNPase I serum autoantibodies in MuS patients and highlight the relevance of this protein as an MuS seric marker.

The comparative analysis of the white matter antigens recognized by serum and CSF IgG from MuS and control patients provided a clear evidence for a compartmentalized autoimmune response in the CSF in MuS but not in control cases. Our study in fact showed that the targets of autoreactive serum MuS IgG were substantially different from those recognized by CSF MuS IgG. These results, together with the recent anatomical and functional evidence of autonomous liquoral immune response (44), highlight the limit of studies assessing the presence of anti-myelin antibodies in the serum of MuS patients. In fact, at variance with serum autoreactivity,

FIG. 4. **Cellular distribution of transketolase in normal human CNS and MuS lesions.** Immunoperoxidase with anti-TK antiserum on paraffin sections from normal human CNS at bulbar (*A* and *B*) and cerebral (*D*) levels showed positive signals on numerous cells in the white matter and ependyma; subpopulations of neurons are negative for TK (*A*), whereas others are clearly immunostained (*D*). *B*, at higher magnification, TK immunoreactivity in the white matter is evident on small, round cells morphologically resembling oligodendrocytes. *C*, the signal is abolished when the primary antibody is omitted. *E*, double immunofluorescence with anti-CNPase monoclonal antibody (E_1, *green*) and anti-TK antiserum (E_2, *red*; E_3, merge image) clearly shows that the majority of mature oligodendrocytes express high levels of TK in their cytoplasm. Shown are consecutive sections immunostained with MBP (*F*) and TK (*G*) showing the edge of chronic MuS lesion; TK immunoreactivity is visible in MBP-positive areas on the *left* but extended also in the demyelinated area. Double immunofluorescence pictures taken at the edge of chronic MuS lesions from frozen sections show the distribution of PLP-positive oligodendrocytes (*green*; H_1) and TK (H_2) with TK-positive cells present in normally myelinated areas and extending also on non-myelinating cells at the lesion border (H_3). Original magnification is as follows: *A*, 150×; *B* and *C*, 400×; *D*, 275×; *E*, 750×; *F* and *G*, 325×; *H*, 400×.

which was mostly directed to cytoskeletal proteins, the majority of MuS CSF had IgG autoantibodies to non-myelin oligodendroglial antigens. Among these, 50% of MuS CSF samples displayed IgG reactivity to a specific isoform of TK, a novel finding that may provide important information for the pathogenesis of MuS. In fact, a reduced activity (because of thiamine deficiency) of this highly conserved enzyme of the PPP has been causally associated with Wernicke-Korsakoff syndrome in which demyelination, neurodegeneration, and blood-brain barrier damage are prominent pathological features (45). Interestingly among the thiamine-dependent enzymes, the demyelinating features observed in such syndrome have been ascribed to TK deficiency because of its role in fatty acid biosynthesis (46). As described previously in rodent CNS (31), we indeed found that TK was mainly expressed by oligodendrocytes in normal conditions; in addition, we found that TK was also present in oligodendrocytes in normal appearing white matter around MuS plaques, whereas at the lesion border TK was expressed by oligodendrocyte precursors, reactive astrocytes, and monocyte/macrophages. This finding is probably related to the fundamental role played by the PPP in the biosynthesis of nucleic acids and lipids during brain development and remyelination (45). Whereas under normal conditions the glucose is minimally metabolized through the PPP in the brain, at times of rapid myelination up to 60% of the cerebral glucose is metabolized via the PPP because of the high rate of lipid biosynthesis (47). In addition to its support to oligodendroglial biology, TK and the PPP may also be relevant to MuS pathogenesis because of their antioxidant role exerted either in oligodendrocytes or neurons (48). Concomitantly to an oxidative stress due to autoimmune-mediated TK deficiency, our results indicate that the autoimmune response may also contribute to axonal/neuronal damage present in MuS (15, 16). In fact, IgG autoreactivity in MuS CSF was directed to a restricted number of neuronal/axonal molecules, and the presence of autoantibodies to specific isoforms of sirtuin 2, radixin, and actin-interacting protein 1 was preferentially found in SP MuS patients, who showed prominent neurodegeneration and irreversible neurological deficits. The small group investigated prevents any clear conclusion about the pathogenic role of such anti-cytoskeletal IgG in axonal degeneration in SP MuS, and we cannot exclude that anti-cytoskeletal antibodies may be a consequence of the axonal damage. However, the preferential CSF antineuronal autoreactivity in SP MuS patients and the lack of association between the number of neuronal antigens recognized by CSF IgG and the disease duration of MuS patients speaks in favor of a pathogenic role for these autoantibodies. For this purpose, additional information from longitudinal studies on a larger group of patients as well as investigations on early stages of MuS are needed.

Taken together, our approach shows that, within a complex array of CNS autoreactivity, MuS patients displayed serum and CSF autoantibodies to a restricted number of antigens localized in oligodendrocytes and neurons/cytoskeleton. Rather than exploring the presence of IgG to single myelin proteins, our results suggest that the simultaneous investigation of multiple antigens may provide a more complete diagnostic and prognostic picture in MuS patients. Exploring the role of non-conventional, non-myelin antigens may add in the near future new and relevant information on MuS pathogenesis, and among these, TK may emerge as a target of CSF autoimmune response.

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